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Journal of Clinical Virology 134 (2021) 104691

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

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https://doi.org/10.1016/j.jcv.2020.104691

Available online 18 November 2020

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ARTICLE INFO

Keywords:
Viral metagenomics
High-throughput sequencing
Next-generation sequencing
Wet lab
Recommendations

ABSTRACT

Metagenomic high-throughput sequencing (mHTS) is a hypothesis-free, universal pathogen detection technique for determination of the DNA/RNA sequences in a variety of sample types and infectious syndromes. mHTS is still in its early stages of translating into clinical application. To support the development, implementation and standardization of mHTS procedures for virus diagnostics, the European Society for Clinical Virology (ESCV) Network on Next-Generation Sequencing (ENNGS) has been established. The aim of ENNGS is to bring together professionals involved in mHTS for viral diagnostics to share methodologies and experiences, and to develop application recommendations. This manuscript aims to provide practical recommendations for the wet lab procedures necessary for implementation of mHTS for virus diagnostics and to give recommendations for development and validation of laboratory methods, including mHTS quality assurance, control and quality assessment protocols.

1. Introduction

Metagenomic high-throughput sequencing (mHTS) is a hypothesis-free, universal pathogen detection technique for the determination of DNA/RNA sequences in a variety of clinical sample types and infectious syndromes. mHTS provides the most comprehensive untargeted approach for the detection of all viruses in a single assay. This approach is suited for identification of any viral pathogen, but particularly for: (i) distinguishing viruses with similar symptom profiles that, in a classic diagnostic laboratory, would therefore require a high number of targeted molecular single-plex assays, (ii) identifying pathogens that not have been associated with a particular symptom profile before, such as astrovirus encephalitis; (iii) discovering novel pathogens which would remain undetectable by target-based methods [1,2]; and (iv) accurate detection and study of RNA viruses with high levels of genetic diversity and therefor often refractory to reliable target-specific diagnostics.

Recently, it has been shown that mHTS workflows allow for identification of pathogens within a clinically relevant timeframe [3]. Furthermore, mHTS allows the simultaneous characterization of complete genome sequences, virulence factors, resistance and epidemiological markers [4]. Despite these clear advantages, mHTS is still in its early stages of translation into clinical application. The current clinical applications of mHTS have focused on patients with encephalitis [5], while research applications are much more common. These include the recent rapid and impactful metagenomic analysis of SARS-CoV-2 [2]. One of the challenges in clinical use of mHTS is the current lack of standardization of mHTS to ensure sensitive and specific pathogen detection. The development of guidelines and recommendations on mHTS methods and workflows will assist the implementation of mHTS in diagnostic laboratories, ensuring the validity of results that will affect patient management.

To support the development and implementation of mHTS procedures for virus diagnostics, a network has been established under the auspices of the European Society for Clinical Virology (ESCV): the ESCV Network on Next-Generation Sequencing (ENNGS). The aim of this network is to bring together professionals involved in mHTS for viral diagnostics and to share methodologies and experiences, and to develop recommendations for the use of mHTS in clinical laboratories.

2. Aim and scope

This review aims to give recommendations for the implementation and validation of laboratory methods for viral mHTS, including quality control (QC) and quality assessment (QA) protocols, but excluding the bioinformatic part of the process, which warrants separate discussion (Part II) outside the scope of the current review. We aim to provide practical recommendations for the pre-analytic and analytic steps for successful implementation of mHTS procedures in viral diagnostic laboratories.

3. Recommendations

3.1. Facility requirements and equipment

3.1.1. Organization of laboratory space

Organization of laboratory space and the diagnostic workflow are vital for the implementation of mHTS due to the unbiased nature of mHTS and the associated increased risk of detection and thus interference of cross-contaminants [6,7]. A common mHTS wet lab workflow consists of five steps, i) reagent preparation, ii) sample preparation and nucleic acid extraction, iii) mHTS library preparation, iv) PCR amplification of the library (adapter extension), and v) product analysis and sequencing (Fig. 1). Additional enrichment procedures may be introduced before and after library preparation. Special emphasis with regard to the risk of cross-contamination in mHTS lies on the steps before adapter ligation. A separate preparation area and lab equipment (e.g. pipettes) is required for diagnostic mNGS and workflows that involve high titre pathogens, such as cultivated and PCR-preamplified samples (e.g. for typing) (**Recommendations 1–4,** Table 1). Separate laboratory spaces for mHTS sample processing may be considered, including a safety cabinet with exclusive use for mHTS.

3.1.2. NGS instruments: use of local and core facilities

mHTS is usually performed on medium and high output platforms, such as the Illumina MiSeq, NextSeq, HiSeq, Novaseq, and Ion S5 (Table 2) systems due to the high number of reads required (>10 million reads per sample) given the generally low proportion of viral reads in clinical samples [8,9]. HTS equipment with smaller sequencing capacity, such as the iSeq, MiniSeq, and MinION [10] are generally used for targeted genomic analysis of a small number of samples, selected microorganisms and fieldwork [11] (**Recommendations 5-6**). Mid-size sequencers like the MiSeq and Ion S5 systems offer the advantage of more frequent runs with only a few samples, however, at higher cost. To make efficient use of high-throughput sequencing instruments within reasonable turn-around-time a shared use with other diagnostic purposes, i.e. molecular pathology or haematology, may be considered.

One option for smaller clinical laboratories is to outsource (parts of) the mHTS workflow to a third-party service provider. Outsourcing of parts of the process may be carefully considered with attention to quality, safety, transparency, and flexibility in case of desired adaptations of the protocol (**Recommendation 7**). Given the contamination issues relating to sample preparation for mHTS, separation of mHTS preps from other library preps performed at third-party providers should be warranted and attention should be paid to carry-over from other samples and runs. Index hopping: misassignment of barcodes between multiplexed Illumina libraries can occur in proportions relevant for pathogen detection even with dual indexing, and this risk should be managed.

Examples of commercial providers for mHTS with and without analysis pipelines are listed in Table 3. Distinction is made between providers of full mHTS service (sample-to-result), commercial kits including analysis software for use in local laboratories, outsourcing of sequencing, and outsourcing of bioinformatic analysis (Part II). Specific attention should be payed to the management and storage of human genomic sequences by third parties, which should be in line with (inter) national regulations.

4. Assay design and development

4.1. Nucleic acid (NA) extraction

The optimal method for NA isolation from clinical samples for mHTS is dependent on the sample type and can be different from those optimized for PCR. DNA and RNA can be co-extracted or isolated separately, and the suitable method should be validated separately for each aim (**Recommendation 8**). For example, while total NA extraction and RNA extraction methods preceding PCR commonly result in comparable quantification cycle (Cq) values in real-time PCR, sequencing of total NA generally results in lower coverage of RNA virus genomes compared to sequencing of samples extracted using specific RNA extraction methods [12]. RNA can be also be obtained by DNase treatment of total NA. The use of high concentrations of carrier RNA should be avoided for RNA mHTS **(Recommendation 9)** as it will be sequenced along with the sample RNA, and thus may affect the ability to detect low level pathogens.

4.2. Host NA depletion and viral enrichment

The paucity of viral NA among the rich background of other sequences may necessitate depletion of host (or bacterial) sequences and/or enrichment of viral sequences either i) prior to NA extraction (*pre-extraction*), by removing whole cells or purifying viral particles, or ii) after extraction, on NA (*post-extraction*). In general, depletion and enrichment protocols affect the unbiased nature of the approach and increase the risk of selective exclusion of (specific) viral sequences.

i) *Pre-extraction*, depletion of whole human cells can be achieved by centrifugation to pellet human cells, filtration to remove human and bacterial cells, treatment of intact cells with a surfactant like saponin [13], cell lysis followed by propidium monoazide treatment [14], or nuclease treatment to remove free non-encapsulated RNA and DNA from the sample. However, pre-extraction depletion of host cells has been reported to be disadvantageous in clinical samples, given the exclusion of intracellular viral particles or NA [15]. For example, more reads from respiratory viruses were found in respiratory samples without enrichment as compared to virus enrichment by cellular filtration [16]. Moreover, pre-extraction viral enrichment protocols are not easily automated and negatively affect the turn-around-time. Instead, the number of specific sequences obtained from clinical samples with low non-enriched target virus concentration can be increased by increasing the number of total sequence reads per sample and such protocols without complicated sample pretreatment can be automated [8].

ii) *Post-extraction*; host NA depletion can be achieved by selective removal of CpG methylated sequences or selective methylation-dependent cleavage of DNA [17] for DNA mHTS, and, by removal of ribosomal RNA of human or bacterial origin, for RNA mHTS. CpG sequences of certain DNA viruses can be methylated, but almost exclusively during latency when integrated in the human genome (e.g. adenoviruses, gammaherpesviruses, papillomaviruses, polyomaviruses) [18]. Removal of ribosomal RNA preceding transcriptome sequencing is commonly performed by poly(A)-mRNA selection. The mRNA of eukaryotic viruses is usually poly(A) tailed, in addition to the genomic template of some viruses (e.g. picornaviruses) [19,20]. Some viruses

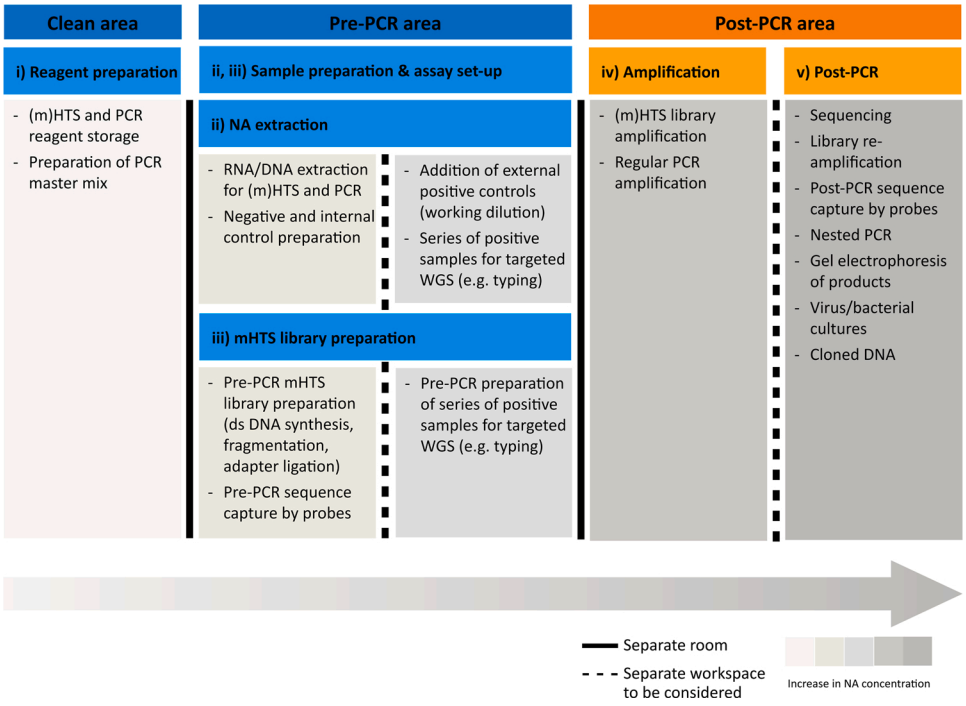

Fig. 1. Schematic overview of the unidirectional workflow of molecular diagnostics including the recommendations for mHTS. NA; nucleic acid.

Table 1
Recommendations for the use of metagenomic sequencing for universal virus diagnostics.

Process step (paragraph)		Recommendation
Facility/floor plan (3.1.1)	1	Physical separation of reagent preparation, pre-amplification and post-amplification library preparation.
	2	Dedicated materials and reagents for each process (sample processing, library preparation, post-library preparation).
	3	Physical separation of metagenomic library preparation from sample preparation of series of positive samples (e.g. for typing), e.g. by using a dedicated biosafety cabinet (BSC) with restricted use for metagenomic workflows.
	4	Extensive cleaning of materials and surfaces with 10% sodium hypochlorite and/or ammonium compound before and after processing, more frequently than regularly performed for molecular assays.
HTS platform (3.1.2)	5	Choice depending on the application and intended use (metagenomics, whole genome sequencing, fieldwork).
	6	Restrict low output sequencers use for a limited number of specimens (due to their lower throughput and multiplexing and deep sequencing capacity).
	7	Consider the number of samples per run in relation with batch-wise sequencing and consequences for turn-around-time. Outsourcing of parts of the process may be carefully considered with attention to quality, safety, transparency and flexibility to desired adaptations of the protocol.
Assay design and development (4)	8	DNA and RNA can be co-extracted or isolated separately, with impact on the mHTS results (sensitivity, coverage), and separate protocols should be validated individually.
	9	Avoid the use of high concentrations of carrier RNA during extraction for RNA mHTS.
	10	Advantages of target enrichment should be weighed against the potential bias introduced by the specific protocol.
	11	SISPA and MDA should not be used when performing viral metagenomics aiming at quantification of viral species, since this may result in over- and underrepresentation of the true proportions for certain viruses.
	12	The minimum number of post-ligation amplification cycles should be used, in order to minimize amplification bias.
	13	The library size distribution should be checked for the expected fragment size, to discard degraded libraries (excess short fragments) or incomplete fragmentation (excess long fragments). Accurate library quantitation ensures adequate library pooling in the sequencing run.
	14	A no-template control that will undergo all steps from sample extraction to sequencing should be used in every individual sequencing run.
	15	More upfront negative controls are recommended to identify sources of potential contamination, such as a library preparation buffer and a pathogen-negative sequence controls (e.g. phage lambda prepared with different reagents).
	16	To control for the success of NA extraction, preparation and sequencing, clinical samples should be spiked with encapsidated RNA or DNA viruses that do not infect humans (vertebrates), e.g. bacteriophages.
Validation & accreditation (5, 6)	17	The following wet lab parameters in the validation process should be included in the validation: sample type, sample volume, extraction protocol, library preperation protocol.
	18	The following sequencing parameters should be included in the validation process: precision, accuracy of sequence output, sequence depth, analytical sensitivity, specificity, limit of detection.
	19	Result interpretation: a cut-off for defining a positive result (read count, coverage) should be determined based on validation data, e.g. comparison with PCR results, using prototype viruses. For defining a positive result, use a threshold of three distinctly covered genome regions after background subtraction based on negative controls.
	20	An external quality assessment programs (EQA) should be adhered to evaluate the performance of metagenomics protocols applied in diagnostic settings, assessing both qualitative (correct pathogen detection) and quantitative characteristics (target read numbers).

initiate translation in the absence of poly(A) tail by using functional analogues (e.g. hepatitis C viruses, rotaviruses [21,22]) and non-replicative viruses may be missed when using this type of selection method. Alternative methods for removal of ribosomal RNA are hybridization to ribosomal oligo probes, and targeted amplification by using 'random' hexamer primers with a decreased affinity for rRNA during first strand cDNA synthesis (see below).

Metagenomic libraries can also be enriched for viral sequences after extraction and reverse transcription steps with capture probe enrichment methods, which are based on hybridisation to a wide set of sequences specific for one or all known vertebrate viruses [23,24]. This strategy may distort the ratios between viruses, but will allow for detection of novel viruses up to a certain degree of nucleotide identity [1]. The significant improvement in sensitivity [23–25] is an advantage for application in clinical virus diagnostics (**Recommendation 10**).

4.3. Double-stranded cDNA synthesis

Viral metagenomics is exceptional among HTS approaches in that the targeted genomes consist of both DNA and RNA. To this end, extracted NA are usually processed separately to generate specific DNA and RNA libraries. Current commercial DNA and RNA library preparation kits require two ends of double-stranded DNA in order to ligate adapters by T4 DNA ligase or transposase. Recently, protocols for direct RNA sequencing of viral RNA have been developed using the Oxford Nanopore Technologies [26]. Double-stranded DNA is usually synthesized using random primers in two subsequent steps: the first and second strand synthesis. As described above, reduction of cDNA synthesis of ribosomal RNA can, at this stage, be achieved by using hexamer primers with reduced affinity for ribosomal RNA [27].

Subsequent procedures to enrich the synthesized double-stranded DNA such as sequence-independent, single-primer amplification (SISPA) [28] and multiple displacement amplification (MDA) using high-fidelity Phi29 polymerase are prone to selective, biased amplification [29]. This unusually high degree of amplification (>30 cycles) of the source material may result in distortion of the viral population ratios and quantitative comparison of viral species. To prevent selective amplification or bias resulting in possible overrepresentation of certain viruses, SISPA and MDA are not recommended for comparison of viral population proportions [30] (**Recommendation 11**). Enrichment for specific viral targets using spiked primers during reverse transcription has been suggested to increase sensitivity, although this method is also biased [31].

4.4. Sequencing library preparation

There are two major strategies for generating sequencing libraries: i) "tagmentation" where enzymatic fragmentation of dsDNA and ligation of adaptors are performed simultaneously (Nextera XT workflow by Illumina) or sequentially (VIDISCA-NGS [32]), and ii) physical

(mechanical or biochemical) shearing followed by DNA fragment end polishing, A-tailing, and finally ligation tagging have been implemented by several workflows. For low-abundance clinical samples, amplification after adapter ligation (12–16 cycles) is needed. Quantitative bias is introduced during post-ligation amplification [33] and even though the clinical implications for qualitative virus detection remain uncertain, it is recommended not to increase the number of post-ligation amplification cycles (**Recommendation 12**). Recently, a number of commercial library kits have become available supporting very low DNA input (as low as 1−5 ng).

The prepared libraries are checked for integrity, size distribution and quantity, and equalized in order to provide comparable counts of total reads per sample during sequencing. It must be noted that this will not result in comparable counts of viral reads given the differences in human and bacterial background reads. Deciding whether or not to sequence a library is a critical step and can change the time to results by a couple of days if a poor quality library is initially sequenced. Important parameters to assess good library quality for clinical testing include library size and concentration, measured by fragment analyzers and/or qPCR. The library size distribution should be checked for ideally a single peak around the expected fragment size, to discard degraded libraries or incomplete fragmentation. Accurate library quantitation is fundamental to normalise library pooling in the sequencing run (**Recommendation 13**).

4.5. Quality controls

Since mHTS will allow the detection of NA contamination from reagents used in sample treatment, library preparation, and sequencing [34–36], a no-template control that will undergo all steps of the workflow in parallel to the sample needs to be included in every individual sequencing run (**Recommendation 14**). This so-called "kitome control" can be used to eliminate the contaminating reads from the patient samples, either manually or by means of automated scripts such as Recentrifuge [37] or Decontam [38]. The use of other upfront negative controls is also recommended to identify sources of potential contamination, such as a library preparation buffer and a pathogen-negative sequence control (e.g. phage lambda prepared with other reagents than the patient sample) (**Recommendation 15**). The use of pathogen-negative human samples as negative controls is less recommended given the variability in human background genome per sample and type of material.

To control the success of NA isolation, library preparation and sequencing in routine diagnostics, spiking of clinical samples with encapsidated RNA and DNA viruses that are not found in humans (vertebrates) are recommended, such as baculoviruses, phocine herpesvirus; tobacco mosaic virus, phages with DNA or RNA genome (MS2 coliphage, Enterobacteria phage T1) [39], or virus-like particles containing non-infectious NA, i.e. Armored RNA (Ambion, Asuragen) (**Recommendation 16**). The amount of internal control added should be optimized to avoid either dropout or competition with pathogenic sequences [40]. The amount of host RNA and DNA will affect the amount of internal control reads, and thus a sharp acceptance threshold is difficult to determine. However, because of this variability, the proportion of internal control reads detected in a clinical sample can be used to normalise viral loads in mHTS quantitative algorithms [41]. To keep track of the performance of the metagenomic workflow over time, an external positive control panel is recommended periodically.

5. Assay validation

At present it is unclear how mHTS will fit into the new European Union (EU) In Vitro Devices Regulation (IVDR), since further guidance on the interpretation of how laboratory-developed tests (LDTs) are regulated is required (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02017R0745-20170505, www.euivdr.com/). Practice-based guidelines and validation studies on mHTS technologies have been recently published in the fields of oncology [42], and broad pathogen detection [40,43]. Many points regarding these requirements are generally applicable for viral mHTS, including the laboratory and data analysis processes. A comprehensive set of recommendations for validation of viral mHTS assays are summarized here **(Recommendations 17–20)**:

Optimal sample volume. Clinical pathogen mHTS protocols in place regularly use 200–600 µL for serum, plasma, CSF or BAL [5,8,30,40,41,44–46]. The used sample volume is one of the factors determining the limit of detection of the mHTS assay, and a higher sample volume is desirable if available, but avoiding excess human reads.

Reference materials. The detection of a wide range of diverse viruses (DNA/RNA, different genome size, double and single stranded, linear and circular, enveloped/non-enveloped) should be analyzed when validating a viral mHTS protocol. In the absence of virus positive residual clinical material, mock samples in the relevant matrix can be made "in-house" using reference material or obtained commercially [47], for instance from the European virus archive (www.european-virusarchive.com), UK-NIBSC (www.nibsc.org), ATCC (www.lgcstandards-atcc.org), Vircell (www.vircell.com), viral multiplex controls (www.nibsc.org/documents/ifu/15−130-xxx.pdf) or a virome mix (www.lgcstandards-atcc.org/products/all/MSA-2008.aspx), available from UK-NIBSC or ATCC (www.lgcstandards-atcc.org/products/all/MSA-4000.aspx). Reference control material should ideally include multiple viruses with known relative loads to monitor specificity, or separate mixtures with high and low viral concentration to monitor sensitivity.

Precision (repeatability, reproducibility). Replicates (intra-assay) and repeats (inter-assay) of aliquoted clinical samples or spiked

Table 2
Main features of current HTS sequencing platforms and most common applications in virology.

Instrument	Av. read length	Final Error Rate (%)	Output per run (Gb)	Runtime (hr.)	Cost per Gb	Primary Use
Illumina benchtop small scale (iSeq, MiniSeq)	2 × 150−2 × 300 bp	~0.1	1.2 – 7.5	4 -55	$100 - $600	WGS, AVR, quasi-species
Illumina medium and large scale (MiSeq, HiSeq, NovaSeq)	2 × 150 bp	~0.1	15 – 6000	12 -144	$7 - $50	Batched samples Metagenomics
Ion Torrent (Proton, S5)	150 bp	~1	1 – 25	2–4	$20 - $80	Rapid runs, WGS S5: metagenomics
PacBio RSII	20 Kb	~1	0.5 - 1	4	$400	Complete SMS genomes
PacBio Sequel II (HiFi, circular mode)	>50 Kb (9−13 Kb)	~1 ~0.1	160	0.5−6	$45	Complete SMS genomes
Oxford Nanopore MinIon (R9-R10)	>200 Kb	2−13	30	1 min-72	$15 - $60	Real-time testing, in field
Oxford Nanopore GridIon (R9-R10)	>200 Kb	2−13	150	1 min-72	$3 - $20	Simultaneous real-time testing

WGS; whole genome sequencing, AVR; antiviral resistance, SMS; single molecule sequencing.

Table 3
External providers for viral/pathogen metagenomics commercial assays (wet lab). RUO; research use only.

Service offered	Provider	Test	Sample	Certified	Website	Citation
Sample referral	Karius	Karius test	Blood	CLIA lab	www.kariusdx.com	[48]
	Pathoquest	iDTECT™ Dx	Blood	CE-IVD	www.pathoquest.com	[47]
	IDbyDNA	Explify Platform	Respiratory Any (RUO)	CLIA lab	www.idbydna.com	[44]
Library preparation reagents and bioinformatics	ARC-BIO	Gallileo™ Pathogen Solution	Blood (Plasma)	RUO	www.arcbio.com	[42]

(internal/mock) controls should be sequenced overtime to test for assay repeatability and reproducibility throughout the entire mHTS workflow. Sample replicates and repeats may include one DNA and one RNA virus (with known concentration or a dilution series), or multiple viruses combined. A virus negative sample should be included. Finally, a number of 20 mHTS runs has been recently used to monitor for changes before assay implementation [47] (Recommendation 18).

Accuracy of sequence output. Sequencing errors and (RT-)PCR induced errors might not compromise the identification of an infectious agent but they can compromise the identification of specific mutations that code for resistance, virulence or transmissibility, especially within quasispecies [48]. When looking for mutations (i.e. antiviral drug resistance), it is useful to assess the introduction of NA amplification and sequencing errors during the HTS process by validating reference materials: well characterized homogenous NA (plasmids including viral inserts, replicons, viral RNA transcripts [49] or Unique Molecular Identifiers) to determine a threshold for the identification of "true" mutations. Bioinformatic scripts can be helpful to reduce (RT-)PCR induced errors from (m)HTS datasets [50–53], and to adapt correction to the different inherent error rates for each platform. For newest technologies with higher error rates, such as nanopore, including closely related viruses during validation can control assay performance to distinguish particular species from background noise.

Sequencing depth and coverage. Sufficient sequencing depth is important to ensure reliable detection of low-abundancy pathogens and low-frequency variants. The acquired depth depends mainly on the sequencing platform in combination with run-time, but also on library preparation, target enrichment and the expected sequence complexity and on the amount of background (human or bacterial) NA and the degree of multiplexing. The required minimum depth is variable per protocol and should be tested for each sample type during the validation stage. For antiviral drug resistance it is recommended that a given mutation is detected with a 1,000x coverage depth, although this depends on the intrinsic error rate of the sequencing platform used.

For virus detection by mHTS, low horizontal coverage of the genome length but with reads distributed over the genome can represent true positive findings [25], whereas a large number of reads (high coverage depth) aligned at one specific part of the genome can represent a false positive result, or novel far related virus, hindering a black and white threshold for percentage and depth of coverage. Overall, a horizontal genome coverage of at least three distinct genome regions aligned after background subtraction based on negative controls is recommended [25,40] (**Recommendation 19**).

5.1. Analytical sensitivity, specificity and limit of detection (LOD)

For a fair comparison of mHTS with conventional routine testing, the performance can only be analyzed in cases where a respective conventional test (same viral target/direct detection method/similar time point) was performed on the same, fresh or defrosted, sample [54]. This shows the inherent difficulty of validating such a broad test that can theoretically detect any pathogen in a given sample. This is aggravated by the fact that in many clinical scenarios (e.g. meningitis/encephalitis) reference standards are missing. For instance, when comparing results to a non-reference standard, the US FDA recommends in their statistical guidance on reporting results from studies evaluating diagnostic tests, to assess sensitivity and specificity as positive and negative percent agreement, respectively (www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm071287.pdf). Spiked samples or well-characterized samples with known copy numbers of viruses are used to establish sensitivity for a "core" set of the target viruses. LOD can be determined by analyzing serial dilutions of a clinical sample containing a known, quantified pathogen subjected to mHTS, or using a set of calibrated internal controls [40]. Cultured virus is not recommended for LOD testing, as cultured viruses may not represent viruses or viral nucleic acid in clinical samples (e.g. herpesviruses [32]). To determine the LOD, cut-off thresholds need to be defined on coverage and sequence depth that are used in decision making always in the context of sample composition (e.g. white blood cell count, grams of tissue), because host nucleic acid burden can quickly change this LOD. The number of pathogen reads can be normalized to those obtained from internal standards [40] and validation data can be obtained by comparing to PCR results using samples with prototype pathogens (e.g. DNA and RNA viruses, double-stranded and single-stranded viruses, circular and linear, enveloped and non-enveloped viruses) **(Recommendation 19).**

5.2. Proficiency testing and external quality assessment (EQA)

EQA of mHTS methods for viral pathogen detection may address the following qualitative characteristics: i) correct pathogen detection at the species level or deeper, ii) quantitative characteristics (e.g. target read numbers) and iii) logistic performance (turn-around-time within a clinically relevant time frame) (**Recommendation 20**). QCMD (www.qcmd.org) aims to launch a viral mHTS metagenomics EQA program by the end of 2020, unaccredited interlaboratory exchange for EQA is second best in complying to the guidelines. Implementation of mHTS in ISO1589 accredited laboratories and the upcoming new in vitro diagnostics regulation raises several questions on for example the extensity of the validation when considering all possible targets. These regulations may result in more frequent and extensive assessment of mHTS protocols both by manufacturers and diagnostic laboratories, potentially leading to more standardization of mHTS protocols, validation requirements, and performance characteristics. Some manufacturers of mHTS library preparation kits and software have requested CE-IVD marking restricted to a limited panel of pathogens that has been validated and compared with conventional, usually molecular assays. Questions are raised when considering the assay performance of detecting micro-organisms that are less easily compared with conventional diagnostic methods, such as cultivation, or because the micro-organism is not tested for at all conventionally, such as the entire population of viruses present in a particular sample, the virome. The above described recommendation on the use of prototype viruses may be a practical consideration (see Recommendation 19).

6. Ethical considerations

When an assay is launched for clinical use, medically important and unimportant findings, as well as findings putatively important have to be considered. Along with accumulating data from research, currently irrelevant findings may become relevant in the future, e.g. if a new disease association is established or if a new drug launched on the market. Thus, storing all sequence information for future use may be justified however subject to (inter)national legislation and is outside the

scope of the current manuscript.

With a potent method such as mHTS, incidental microbiological findings are to be expected up-front. The clinician has to be aware of such a possibility and has to be prepared to explain the impact of such findings to the patient. Some findings may be unrelated to the patient's illness but may be significant for their health (e.g. finding an unexpected HIV, HBV or HCV infection). How to deal with such findings should be properly documented before launching an mHTS assay. However, stochastic findings should not unnecessarily complicate result interpretation. How to proceed with sequence reads of human host background has to be considered as well, as they contain even more sensitive information, and this will be addressed in Part II of the Recommendations. With nanopore technology's, selectively excluding sequencing of human background DNA reads could be an option [55].

7. Conclusions

For some clinical syndromes, such as encephalitis, there is a need to extend the diagnostic portfolio with mHTS. For many others, due to the cost and turn-around-time constraints, none of the currently available mHTS methods seem capable of completely replacing conventional diagnostic testing in the near future. Nonetheless, the recommendations provided here are intended to guide laboratories on the implementation of mHTS for Clinical and Public Health Virology diagnostic workflows. Technical, procedural and financial parameters will develop rapidly, and it is anticipated that these future developments will support the progressive and broad introduction of metagenomic sequencing into Clinical and Public Health diagnostic laboratories.

CRediT authorship contribution statement

F. Xavier López-Labrador: Conceptualization, Writing - original draft. **Julianne R. Brown:** Writing - review & editing. **Nicole Fischer:** Writing - review & editing. **Heli Harvala:** Writing - review & editing. **Sander Van Boheemen:** Writing - review & editing. **Ondrej Cinek:** Writing - original draft, Writing - review & editing. **Arzu Sayiner:** Writing - original draft. **Tina Vasehus Madsen:** Writing - original draft. **Eeva Auvinen:** Writing - original draft. **Verena Kufner:** Writing - original draft. **Michael Huber:** Writing - original draft, Writing - review & editing. **Christophe Rodriguez:** Writing - review & editing, Writing - review & editing. **Marcel Jonges:** Writing - original draft. **Mario Hönemann:** . **Petri Susi:** Writing - original draft, Writing - review & editing. **Hugo Sousa:** Writing - review & editing. **Paul E. Klapper:** Writing - review & editing. **Alba Pérez-Cataluña:** Writing - review & editing. **Marta Hernandez:** Writing - original draft, Writing - review & editing. **Richard Molenkamp:** Writing - review & editing. **Lia van der Hoek:** Writing - review & editing. **Rob Schuurman:** Writing - review & editing. **Natacha Couto:** Writing - review & editing. **Karoline Leuzinger:** Writing - review & editing. **Peter Simmonds:** Writing - original draft. **Martin Beer:** Writing - review & editing. **Dirk Höper:** . **Sergio Kamminga:** Writing - original draft. **Mariet C.W. Feltkamp:** Writing - review & editing. **Jesús Rodríguez-Díaz:** Writing - review & editing. **Els Keyaerts:** Writing - original draft. **Xiaohui Chen Nielsen:** Writing - original draft. **Elisabeth Puchhammer-Stöckl:** Writing - original draft. **Aloys C.M. Kroes:** Writing - original draft. **Javier Buesa:** Writing - review & editing. **Judy Breuer:** Writing - review & editing. **Eric C.J. Claas:** Conceptualization, Writing - original draft. **Jutte J.C. de Vries:** Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors report no declarations of interest.

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